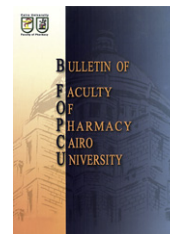




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**ORIGINAL ARTICLE**

Simultaneous HPLC–UV analysis of telmisartan and hydrochlorothiazide in human plasma

Ismail Salama *

Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Suez Canal University, Ismailia 41522, Egypt

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Abstract A specific, sensitive and rapid method based on high performance liquid chromatography (HPLC) was developed for the simultaneous determination of telmisartan (TELM) and hydrochlorothiazide (HCT) in human plasma using indapamide as internal standard. The method utilizes proteins precipitation with acetonitrile as only sample preparation prior to RP-HPLC.

The analytes were chromatographed on shim-pack cyanopropyl column in isocratic elution with methanol: 10 mM ammonium acetate solution (pH 6.0) (35:65 v/v) as mobile phase at a flow rate of 1 ml/min and the wavelength of detection was 270 nm.

The method was validated over the concentration range of 1–10 µg ml⁻¹ for TELM and 0.31–3.12 µg ml⁻¹ for HCT in human plasma.

Inter- and intra-run precision of TELM and HCT were less than 3.60% and the accuracy was less than 1.868%. The linearity, recovery, matrix effect and stability were validated for TELM/HCT in human plasma.

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1. Introduction

Telmisartan (TELM), 4'-[(1,4'-dimethyl-2'-propyl[2,6'-bi-1H-benzimidazol]-1'-yl)methyl][1,1'-biphenyl]-2-carboxylic acid¹; It is a newly developed drug, which is angiotensin II receptor antagonist, used in the treatment of hypertension. Telmisartan is not official in IP, BP, and USP. Hydrochlorothiazide is chemically 6-chloro-3,4-dihydro-2H-1,2,4-benzothiadiazine-7-sulphonamide-1,1-dioxide. It is official in IP'96,² BP 2004³ and USP 2006.⁴ The two drugs are of limited metabolism and eliminated unchanged from human body.⁵

Detailed survey of literature of telmisartan revealed several methods for its determination in pharmaceutical formulations like, electrochemical^{6,7} and HPLC^{8,9} also telmisartan was determined in human plasma^{10–12} and in urine.¹³ Similarly, a

* Corresponding author. Mobile: +20 0180327695; fax: +20 0643230741.

E-mail address: ismailpharm@yahoo.com

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survey of literature of hydrochlorothiazide revealed that it has been measured in biological fluid both by HPLC^{14–16} and electrochemical¹⁷ methods. UV spectrophotometric, TLC, and spectrofluorimetric methods were found to be reported for the determination of telmisartan and hydrochlorothiazide in a combined dosage form.¹⁸ Two HPLC methods for determination of telmisartan and hydrochlorothiazide in pharmaceutical preparation^{19,20} were also reported. Liquid chromatographic–tandem mass spectrometric method for the simultaneous quantitation of telmisartan and hydrochlorothiazide in human plasma²¹ was also reported; this method required long time for preparation of samples, relied on tedious liquid–liquid extraction procedure, used complicated gradient elution and expensive equipments.

Therefore, it was desirable to develop simple, accurate, cheap and fast procedure that could be applied for the simultaneous determination of telmisartan and hydrochlorothiazide in plasma after protein precipitation as the only sample preparation step involved prior to reversed phase-HPLC.

The present work presents simple, rapid and sensitive economical method for determination of TELM and HCT in human plasma compared to published methods. A good separation of the analytes of this combination was achieved by using a mobile phase containing ammonium acetate and methanol as mobile phase. The proposed method is rapid, less expensive, and is successfully applied for the simultaneous determination of telmisartan and hydrochlorothiazide in human plasma; the method utilizes proteins precipitation with acetonitrile as only sample preparation prior to RP-HPLC.

2. Material and methods

2.1. Chemicals and reagents

Methanol and acetonitrile were of HPLC grade (Riedel-de Haen laboratory chemicals, Germany). Ammonium acetate, acetic acid, and NaOH used were of analytical grade.

The ammonium acetate buffer pH 6.0 was prepared by dissolving 0.77 g in liter of distilled water and pH was adjusted to 6.0 using acetic acid and 0.1 N NaOH.

Telmisartan and hydrochlorothiazide were kindly supplied by alkan Pharma Cairo, Egypt) and certified to contain 99.7% and 99.8%, respectively, and used without further purification.

Biocardis tablets were manufactured under license of Biopharma. Each tablet contains 80 mg TELM, 12.5 mg HCT.

Drug-free human plasma was obtained from hospital blood bank.

2.2. Calibration standards (CS) and quality control (QC) samples in human plasma

Stock solutions were prepared by dissolving each of TELM, HCT and indapamide in methanol to obtain concentration of 1 mg ml⁻¹ for each. The solution was prepared by dissolving 100 mg of each drug in sufficient amount of methanol and the volume was completed to 100 ml volumetric flask with the same solvent.

Working standard solutions were prepared by transferring different volumes 0.5–5 ml of stock TELM and 0.155–1.55 ml of stock HCT to 10 ml volumetric flask and the volume

is completed with methanol. Volumes of 20 µl of working standard solutions and 20 µl of I.S. stock solution were added to 960 µl of drug-free human plasma to obtain drug concentration levels of 1–10 µg ml⁻¹ for TELM and 0.31–3.12 µg ml⁻¹ for HCT.

Quality control (QC) samples were prepared separately and pooled at three different concentration levels (1, 5, and 10 µg ml⁻¹ for TELM and 0.62, 2.17, and 3.12 for HCT) as low, medium and high, respectively. A calibration curve was constructed from a blank sample (a plasma sample processed without I.S.), a zero sample (a plasma processed with the I.S.) and non-zero samples covering the total range 1–10 µg ml⁻¹ for TELM and 0.31–3.12 µg ml⁻¹ for HCT.

2.3. Plasma sample preparation

The stored plasma samples were allowed to thaw at room temperature before processing. The plasma samples were centrifuged at 4000 rpm for 10 min, an aliquot (0.96 ml) was pipetted into a 10 ml polypropylene tube and acetonitrile (2.0 ml) was added. The mixture was vortex mixed briefly, and after standing for 5 min at room temperature, the mixture was centrifuged at 4000 rpm for 20 min. the supernatant was carefully transferred into vial and injected into HPLC system.

2.4. Liquid chromatography/UV spectrophotometry

Plasma samples were analyzed using a HPLC (Shimadzu, Kyoto, Japan) instrument equipped with a model series LC-10 ADVP pump, SCL-10 AVP system controller, DGU-12A Degasser, Rheodyne 7725i injector with a 20 µl loop and a SPD-10AVP UV–Vis detector. Separation and quantitation were made on a Shim-pack® cyanopropyl column (250 × 4.6 mm (I.d.)) (5 µ particle size). The detector was set at 270 nm. Data acquisition was performed on class-VP software.

The mobile phase was composed of 10 mM ammonium acetate solution (pH 6.0)–methanol (65:35, v/v). The flow rate was 1 ml min⁻¹. The Injected volume was 20 µl. Detection was performed with UV–Vis detector UV–Vis at λ 270 nm.

2.5. Method validation

The method was validated for selectivity, matrix effect, precision, accuracy, linearity, sensitivity, recovery and stability according to the US Food and Drug Administration (FDA)²² guidelines for the validation of bioanalytical method.

The selectivity of this method was investigated by analyzing six individual human blank plasma samples. Each blank sample was tested for interference using the present analytical method and was compared with spiked sample whose concentration of the analyte was at the LLOQ.

Calibration standard samples in human plasma were prepared for three separate batches. Intra- and inter-batch precision and accuracy were evaluated by measurement of TELM and HCT in plasma in five replicates of QC samples at three different concentrations for three separate batches.

The matrix effect was investigated by comparing the calculated TELM/HCT concentration in spiked QC samples in six biological matrices separately from different drug-free volunteers (A) and in mobile phase (B) at different concentration levels. Similarly, the matrix effect on the I.S. was measured from their peak areas at the working concentration level.

Matrix effect was defined as the concentration or peak ratio ($A/B \times 100$). During the preparation of QCs or blank samples at the same concentration level, each individual's biological matrix was used only once. The inter-subject variability of matrix effect at every concentration level should be less than 15% for acceptable performance.²³

The stabilities of TELM and HCT in biological matrix and working solutions at different storage conditions were evaluated as follows and the results were expressed as percentage recoveries (concentration of sample under different storage condition/theory concentration).

The stabilities of TELM and HCT working solutions were tested for 6 h at ambient temperature.

The stability of the studied drugs in human plasma was assessed under different study conditions; i.e. standing at ambient temperature over 24 h (Bench top-stability) using QC samples at low, medium and high concentration levels and storing at -20°C for one month (long-term stability). QC samples experiencing three freeze-thaw cycles (freeze–thaw stability) were analyzed together.

3. Results and discussion

To obtain the best chromatographic condition, different columns and mobile phases with different pH and organic modifier were tested to provide sufficient selectivity and sensitivity in short separation time.

The best chromatographic condition take placed on cyanopropyl column with mobile phase consisting of 10 mM ammonium acetate solution (pH 6):methanol (65:35) at flow rate 1 ml min^{-1} and UV detection at 270 nm (Fig. 1).

The influence of both organic modifier concentration and pH were carefully studied. Increasing organic modifier concentration not only improve peak shape and decrease the run time but also decrease the method specificity due to the interference of the HCT peak with endogenous biological substance. Decreasing of organic modifier concentration than 20%

resulted in high specificity with regard to the separation of the studied drug from endogenous biological substances, and more retained of the drug on the column that led to excessive tailing of eluting peaks and long run time.

Variation of pH plays an important role in the separation process, at pH (3.5), TELM become more retained on the column. pH (6.0) was chosen as the optimum value both for resolution of drugs from endogenous biological substances and better peak shape and reasonable run time.

One of the main reasons for using an internal standard is for sample requiring significant pretreatment or preparation (filtration, extraction) result in sample losses.

Properly chosen internal standard can be used to correct sample losses. Indapamide was considered as best (I.S.) applied for determination of TELM and HCT owing to retention characters that not affected seriously by change of pH, so it eluted with reasonable resolution from TELM and HCT, and absorbance characters that show high absorbance at the chosen wave length (270 nm) that increase the sensitivity of the method.

The proposed method succeeds in the determination of the studied mixture in spiked plasma sample and also in real patient plasma sample, 2 h following oral administration of Biocardis tablets (80 mg TELM, 12.5 mg HCT) (Figs. 1 and 2).

4. Methods validation

4.1. Linearity

Linearity was established by analyzing six concentrations of TELM and eight concentrations of HCT ranging between $1\text{--}10$ and $0.31\text{--}3.12\text{ }\mu\text{g ml}^{-1}$, respectively, by plotting the peak area ratio against the corresponding concentration. Linearity of the calibration graphs was validated by the high value of the correlation coefficient (>0.99) and the intercept value; (Table 1). The calibration range for the proposed method was established through considerations of the practical range

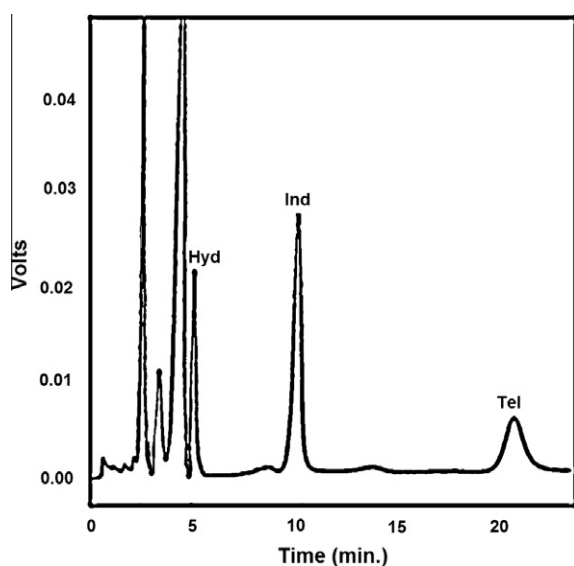


Figure 1 HPLC chromatogram of 20 μL injection of telmisartan (Tel), hydrochlorothiazide (Hyd), and indapamide (Ind) as I.S. spiked in human plasma.

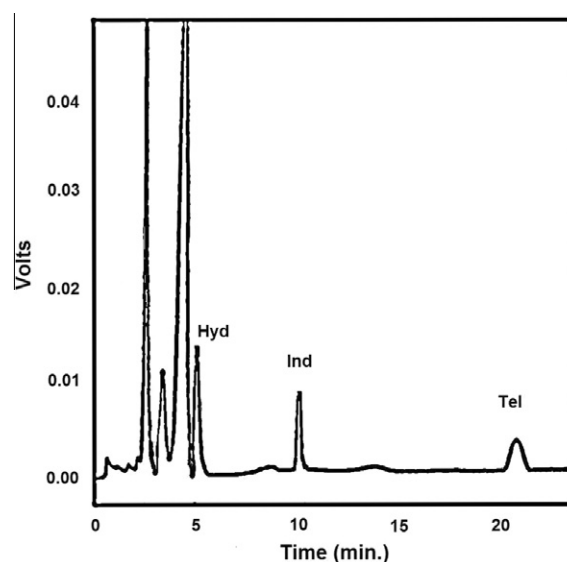


Figure 2 HPLC chromatogram of 20 μL injection of patient plasma sample, 2 h following oral administration of Biocardis tablet (80 mg telmisartan and 12.5 mg hydrochlorothiazide).

Table 1 Assay parameters and regression characteristic of the binary mixture determined by the proposed method.

Parameters	Telmisartan	Hydrochlorothiazide
Linearity range ($\mu\text{g ml}^{-1}$)	1–10	0.31–3.12
Detection limit ($\mu\text{g ml}^{-1}$)	0.054	0.043
Quantitation limit ($\mu\text{g ml}^{-1}$)	0.180	0.140
n	6	6
Slope (b)	0.046	0.529
S.D. of slope	0.001	0.009
R.S.D. of slope	2.170	1.720
Intercept (a)	0.006	0.022
S.D. of intercept	0.005	0.037
Correlation coefficient (r)	0.997	0.999
S.E. of regression	0.004	0.084

Regression equation: $A = a + bc$, where A is the absorbance, a is the intercept, b is the slope and c is the concentration.

required and the concentrations of TELM and HCT present in the pharmaceutical product to give accurate, precise and linear results (Table 1).

4.2. Accuracy

The method was repeated for different concentrations of pure samples (quality control samples) at the low, medium and high

concentration levels, each repeated six times. The concentrations were calculated each from its corresponding regression equation. The recovery percentages and the mean recoveries and the relative error (RE) were then calculated and the results were shown in Table 2.

4.3. Precision

To measure the degree of method repeatability, the quality control samples were analyzed each six times within the same day and in three successive days as shown in Table 2.

4.4. Stability

The stability tests of the analytes were designed to cover expected conditions concerning the handling of clinical samples. The stabilities of the analytes in human plasma were investigated under various storage and processing conditions. The results are summarized in Table 3. The results indicate that TELM and HCT were stable for the entire period of the experiment.

4.5. Selectivity and sensitivity

No endogenous source of interference was observed at the retention times of the analytes. Typical chromatogram

Table 2 Validation results of the proposed method.

Analyte	Added conc. ($\mu\text{g/ml}$)	Found conc. ($\mu\text{g/ml}$) (mean \pm S.D.)	Intra-day R.S.D. (%)	Inter-day R.S.D. (%)	R.E. (%)
TEL	1	0.890 \pm 0.015	1.690	2.212	−11.400
	5	4.990 \pm 0.067	1.350	3.510	−0.800
	10	10.100 \pm 0.079	0.789	1.779	0.100
	0.62	0.610 \pm 0.005	0.840	0.270	−4.030
HCT	2.17	2.070 \pm 0.073	3.589	2.973	−6.266
	3.12	3.100 \pm 0.073	2.297	1.407	1.868

^aThe intraday ($n = 3$), average of three different concentrations repeated three times.

^bThe interday ($n = 3$), average of three different concentrations repeated three times.

Table 3 Summary of stability of TELM & HCT in human plasma at varying condition.

Theoretical concentration ($\mu\text{g ml}^{-1}$)						
Telmisartan			Hydrochlorothiazide			
1	5	10	0.62	2.17	3.12	
<i>(a) Three freeze thaw cycles</i>						
Mean	0.983	5.02	10.07	0.595	2.036	3.18
S.D.	0.015	0.10	0.087	0.005	0.072	0.075
RSD (%)	1.53	1.99	0.86	0.84	3.54	2.36
RE (%)	−1.7	0.4	0.7	−4.03	−6.18	1.92
<i>(b) Room temperature for 24 h</i>						
Mean	0.886	4.96	10.01	0.58	2.15	2.98
S.D.	0.015	0.067	0.079	0.01	0.07	0.02
R.S.D. (%)	1.69	1.35	0.789	1.72	3.26	0.67
RE (%)	−11.4	−0.8	0.1	−6.45	−0.92	−4.49
<i>(c) Re-injection after 30 days at -20°C</i>						
Mean	0.93	5.11	10.06	0.59	2.01	3.04
S.D.	0.02	0.135	0.081	0.01	0.05	0.08
R.S.D. (%)	2.15	2.64	0.805	1.69	2.49	2.63
RE (%)	−7	2.2	0.6	−4.84	−7.37	−2.56

Table 4 Statistical analysis of parameters required for system suitability testing of the proposed method.

Parameter	Obtained value			Reference value
	TEL	Ind	HCT	
Resolution (Rs)	4.650		5.000	$R > 0.8$
Relative retention (α)	2.250		2.660	> 1
Tailing factor (T)	1.000	1.000	1.088	$T = 1$ for a typical symmetric peak
Capacity factor (K')	9.000	4.000	1.500	1–10 Acceptable
Number of theoretical plates (N)	2220	3468.750	3468.750	Increases with efficiency of the separation
HETP (cm/plate)	0.011	0.007	0.007	The smaller the value, the higher the column efficiency

obtained from a plasma sample spiked with telmisartan, hydrochlorothiazide, and indapamide is presented in (Fig. 1).

4.6. Determination of limit of quantitation and detection

For determining the limit of detection (LOD) and quantitation (LOQ), the method based on the residual standard deviation of a regression line and slope was adopted.²⁴ To determine the LOD and LOQ, a specific calibration curve was constructed using samples containing the analytes in the range of LOD and LOQ.

The LOD and LOQ for telmisartan and hydrochlorothiazide were estimated at a signal-to-noise ratio of 3:1 and 10:1, respectively. The LODs for Telmisartan and hydrochlorothiazide were 0.018 and 0.022 $\mu\text{g ml}^{-1}$, and the LOQs were 0.052 and 0.068 $\mu\text{g ml}^{-1}$, respectively.

LOD and LOQ were calculated by using following equations.

$$\text{LOD} = C_d \times \text{Syx}/b$$

$$\text{LOQ} = C_q \times \text{Syx}/b$$

where C_d/C_q is coefficient for LOD/LOQ; Syx is residual variance due to regression; b is slope.

4.7. Robustness

The proposed HPLC method proved robustness when i tried to induce minor deliberate changes in the organic strength ($\pm 1.5\%$) and the pH (± 0.1 unit) of the mobile phase where the retention time of the peaks was not significantly affected (± 0.01 min).

4.8. Ruggedness

The proposed HPLC method showed ruggedness when we tried to transfer our model to another lab with another HPLC instrument (Shimadzu, Japan) in another city and running the analysis via another fellow analyst as well, where the same results and retention times were obtained (± 0.01 min) proving no significant lab to lab, instrument to instrument, analyst to analyst and time to time variations and hence ruggedness of the method.

4.9. System suitability studies

The resolution, number of theoretical plates, and peak asymmetry were calculated for the working standard solutions and are as shown in Table 4. The values obtained demon-

strated the suitability of the system for the analysis of these drugs in combination in human plasma.

4.10. Comparison of the proposed method with the reported method

When comparing between the proposed method and the published method for simultaneous determination of the binary mixture²¹ found that:

- The suggested method is highly sensitive (linearity range; 1–10 $\mu\text{g ml}^{-1}$ for TELM and 0.31–3.12 $\mu\text{g ml}^{-1}$ for HCT) when compared to the published method (linearity range; 1.00–600 ng ml^{-1} for both analytes).
- The proposed method utilizes proteins precipitation with acetonitrile as the only sample preparation involved prior to reverse phase-HPLC rather than the other method that depend on liquid–liquid extraction that need long time and effort for sample preparation before injection, and also the possibility of drug lost during preparation is high.
- The proposed method uses isocratic elution which is simple compared to the other method which uses gradient elution, also HPLC/UV is less expensive than LC–MS/MS.
- The published method however has shorter run time and performed pharmacokinetic study.

5. Conclusion

The proposed HPLC method is a suitable technique for simultaneous determination of the binary mixture in human plasma. The method is simple, accurate, and utilizes proteins precipitation with acetonitrile as the only sample preparation prior to RP-HPLC. The rapid, single step, plasma preparation coupled with the simple HPLC–UV isocratic chromatographic apparatus makes the method cost-effective and suitable for analysis of a large number of samples. The method was fully validated to meet the requirements for sensitivity, accuracy and precision from State Food and Drug Administration.

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References

- Budavari S, editor. *Merck*. 13th ed. Whitehouse Station, NJ, USA: Merck & Co Inc; 2001.
- Indian Pharmacopoeia*. New Delhi: The Controller of Publication; 1996.
- British Pharmacopoeia*. International edition. London: HMSO Publication; 2004.
- The United States Pharmacopoeia*. 29th ed. Asian edition. Rockville, MD: USP Convention Inc.; 2006.
- Sweetman SC, editor. *Martindale – the complete drug reference*. 36th ed. London: Pharmaceutical Press; 2009.
- Xu MT, Song JF, Liang YD. Rapid determination of Telmisartan in pharmaceutical preparation and serum by linear sweep polarography. *J Pharm Biomed Anal* 2004;**4**(3):681–7.
- Xu MTT, Song JFF, Li N. Rapid determination of Telmisartan in pharmaceutical and serum by parallel catalytic hydrogen wave method. *Anal Bioanal Chem* 2003;**377**(7–8):1184–9.
- Palled MS, Rajesh PMN, Chatter M, Bhat AR. RP-HPLC determination of Telmisartan in tablet dosage forms. *Ind J Pharm Sci* 2005;**67**(1):108–10.
- Rao RN, Sen S, Nagaraju P, Reddy VS, Krishnamurthy PR, Bhaskar SU. HPLC determination of Telmisartan in bulk and pharmaceutical formulation. *Asian J Chem* 2006;**18**(2):775–82.
- Chen BM, Liang YZ, Wang YL, Deng FL, Zhou P, Guo FQ, et al.. Development and validation of LC–MS method for determination of Telmisartan in human plasma. *Anal Chim Acta* 2005;**540**(2):367–73.
- Shen J, Jiao Z, Li ZD, Shi XJ, Zhong MK. HPLC determination of Telmisartan in humans plasma and its application to pharmacokinetic study. *Pharmazie* 2005;**60**(60):418–20.
- Zhang H, Jiang Y, Wen J, Zhou T, Fan G, Wu Y. Rapid determination of telmisartan in human plasma by HPLC using a monolithic column with fluorescence detection and its application to a bioequivalence study. *J Chromatogr B Analyt Technol Biomed Life Sci* 2009;**29**:3729–33.
- Torrealdy N, Gonzalez L, Alonso RM, Jimenez RM, Lastra EO. Experimental design approach for optimization of HPLC fluorimetric method for quantitation of angiotensin II receptor antagonist Telmisartan in urine. *J Pharm Biomed Anal* 2003;**32**(4–):847–57.
- Zendelovska D, Stafilov T, Milosevski P. Development of solid phase extraction method and its application for determination of hydrochlorothiazide in human plasma using HPLC. *Biomed Chromatogr* 2004;**18**(2):71–6.
- Rajasekhar D, Kumara IJ, Venkateswarlu P. High performance liquid chromatography/negative ion electrospray tandem mass spectrometry method for the measurement of hydrochlorothiazide in human plasma: application to a comparative bioavailability study. *Eur J Mass Spectrom* 2009;**15**(6):715–21.
- Shao QX, Wang KS, Wei Y, Sun YY. HPLC determination of hydrochlorothiazide in human plasma. *Yaowu Fenxi Zazhi* 2010;**30**(3):396–8.
- Razak OA. Electrochemical study of hydrochlorothiazide and its determination in urine and tablet. *J Pharm Biomed Anal* 2004;**34**(2):433–40.
- Lories IB, Samah SA, Laila AF, Heba HR. Application of first derivative, ratio derivative spectrophotometry, TLC densitometry, and spectrofluorimetry for the simultaneous determination of Telmisartan and hydrochlorothiazide in pharmaceutical dosage forms and plasma. *IL Farmaco* 2005;**60**(10):859–67.
- Bhat LR, Godge RK, Vora AT, Damle MC. Validated RP-HPLC method for simultaneous determination of telmisartan and hydrochlorothiazide in pharmaceutical formulation. *J Liq Chromatogr Relat Technol* 2007;**30**(17–20):3059–67.
- Rane VP, Sangshetti JN, Shinde DB. Simultaneous high-performance liquid chromatographic determination of telmisartan and hydrochlorothiazide in pharmaceutical preparation. *J Chromatogr Sci* 2008;**46**(10):887–91.
- Yan TT, Li H, Deng LL, Guo YJ, Yu WS, Fawcett JP, et al.. Liquid chromatographic–tandem mass spectrometric method for the simultaneous quantitation of telmisartan and hydrochlorothiazide in human plasma. *J Pharm Biomed Anal* 2008;**48**(4):1225–9.
- US Department of Health and Human Services Food and Drug Administration; 2001. <<http://www.fda.gov/cder/guidance/index.htm>>.
- Taylor PJ. Matrix effects: the Achilles heel of quantitative high-performance liquid chromatography–electrospray–tandem mass spectrometry. *Clin. Chem.* 2005;**38**:328–34.
- Drug Information Branch (HFD-210). Validation of analytical procedures: methodology, step 4. In: *ICH harmonized tripartite guidelines Q2B*. Rockville, MD: Center for Drug Evaluation and Research; 1996.